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Acute toxicological impacts of copper in mud crab *Scylla serrata* by comprehensive biochemical, enzymatic, genotoxicity and histological assessments

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Abstract

This study comprehensively analyses copper toxicity in *Scylla serrata* (mud crab) at enzymatic, biochemical, genotoxicity and histological assessments. The acute toxicity effects of heavy metal copper exposure on *Scylla serrata* were analysed, and the LC_{50} concentration of copper at 96 h was identified as 0.52mg/L, which indicates moderately stronger sensitivity of *Scylla serrata* to copper. The acute copper exposure to *Scylla serrata* induces oxidative stress eventually activating the antioxidant enzymes as a primary defense. Acute copper exposure leads to DNA fragmentation and induces genotoxicity in *Scylla serrata*, adversely affecting the organism. The copper toxicity poses an increased risk in mud crab culture, leading to reduced survival rates, impaired growth and eventually impacting economic sustainability and consumer health risks. These findings provide a rare comprehensive assessment of acute copper toxicity in *Scylla serrata*, bridging gaps in the existing literature. Our findings highlight potential threats to aquaculture systems, economic sustainability, food safety and urge for stricter monitoring of heavy metal contaminations in aquacultural farms.

1 Introduction

In shellfish aquaculture, especially in crab cultivating ponds, the copper sulphate is commonly used to eliminate filamentous algae and also control pathogens (Tang et al., 2021). Copper is an important element in the molecular structure of respiratory pigment hemocyanin of some crustaceans, and it acts as an enzyme cofactor, participating in several physiological processes of animals (Velayutham and Munusamy, 2016). The element copper has an important role in the crustacean's metabolism. However, copper causes toxic effects to aquatic organisms, including crustaceans, when its internal concentration exceeds the required level (Chavez-Crooker et al., 2003). Copper sulphate pentahydrate is the most regularly used formulation in water bodies. One of the major applications of sulphate in pond management is used as an algicide, fungicide, and herbicide in agriculture and aquaculture, and also as a therapeutic against infections in the fishes, and so on (Tavares-dias, 2021).

Crustaceans are the second largest group among arthropods, and cultivable crustaceans play a significant role in the economy of the global aquaculture industry (Leblanc, 2007). In crustaceans, the trace elements accumulate in their tissues and can move through various biogeochemical cycles and bioaccumulate (Li et al., 2025). The *Scylla serrata* is one of the significant cultivable crustaceans, particularly in Southeast Asia and India, due to its wide popularity as it contains essential nutrients and is rich in protein (Kathirvel M., Kulasekarapandain S., 2004). However, the copper metal pollution in aquaculture systems poses serious threats to mud crab growth and reproduction and potentially leads to economic sustainability and food safety concerns. The *S. serrata* relies on the complex network of biochemical elements such as proteins, amino acids, carbohydrates, lipids, and numerous secondary metabolites to carry out its essential biological functions (Hamid et al., 2024).

The toxic effects of copper in various crab species are reported, including *Carcinus maenus* (Blewett and Wood, 2015)(Niyogi et al., 2016)(Rossbach et al., 2017)(Luo et al., 2020), *Sesarma quadratum* (Santhana

Valarmathi, 2002) *Portunus pelagicus* (Ketpadung et al., 2006) and *Neohelice granulata* (Sabatini et al., 2009), *Callinectus sapidus* (Paganini and Bianchini, 2009)(Martinez et al., 2011)(Guerreiro Gomes et al., 2019), *Eriocheir sinensis* (Machado et al., 2012) (Bu et al., 2022) (Feng et al., 2022), *Eriocheir japonica sinensis* (Tang et al., 2021), *Pachygrapsus marmoratus* (Oliva et al., 2019) and in *Minuca rapax* (Capparelli et al., 2020). Several studies have reported the effect of copper on crabs across various biological and environmental aspects. However, the level of toxicity varies with crab species and environmental factors, including climate change and habitat conditions. Therefore, it is necessary to investigate species-specific heavy metal toxicity. In the current study, we investigate the copper heavy metal toxicity in laboratory conditions using *S. serrata*.

This study provides a multi-level copper toxicity assessment in *S. serrata* integrating biochemical and oxidative stress biomarkers, metabolic enzymes activity, DNA damage (single-gel electrophoresis), and histopathological alterations. To the best of our knowledge, detailed information on the toxic effects of copper in *S. serrata* is very limited. By bridging this knowledge gap, we provide critical insights into the acute copper exposure impacts on the key aquaculture species *S. serrata.*

2 Materials and methods

2.1 Study areas and sampling

The mud crabs *S. serrata* were collected from a local farm in Kozhikode, Kerala. The crabs were identified using taxonomic identification keys in the Marine Species Identification Portal (Forskal, 1775) (Keenan et al., 1998).

2.2 Determination of LC_{50} concentration

A total of 90 healthy male crabs at the intermoult stage (weight ranging from 125-150g and carapace length was 9 ± 2cm) were used to find copper LC_{50} concentrations at 96 h. The crabs were acclimated to laboratory conditions for one week before experiments. Crabs were kept in separate cages (100×60×40 cm), continuously aerated and the water was changed every 12 h to maintain the copper concentrations and to avoid any infections. Water quality parameters such as salinity, temperature, pH, and dissolved oxygen were monitored every 12 h. The values were maintained as salinity 35 ± 2ppm, temperature 27 ± 1ºC, pH as 7 ± 0.5, and the dissolved oxygen as > 6mg/L to assure copper concentrations were stable. The mortality rate of crabs was recorded at 24, 48, 72, and 96 h to measure the LC_{50} concentration of copper to *S. serrata*. Weighing 3.906 g of $CuSO_4 \cdot 5H_2O$ and dissolve it in a volumetric flask, then dilute with distilled water up to the final volume of 1000 mL (1 L). A total of 90 healthy male crabs were used to find copper LC_{50} concentrations at 96 h. Initially, preliminary range finding test was conducted from 0, 0.25, 0.50, 0.75, 1.0, 1.25, 1.50, 1.75 and 2.0 mg/L copper concentration. This screening reveals 100% mortality above the 1 mg/L. based on these results, the definitive LC_{50} assay was performed for the copper concentrations within the 1mg/L specifically 0.001, 0.01, 0.1, 1.0 mg/L. The mortality data were analysed using Finney's probit analysis method in IBM SPSS (Version 20) to determine the LC_{50} at 96 h

(Finney et al., 1947). To confirm the LC50 a subsequent mortality test (in laboratory condition) was performed using a subsequent mortality test was performed using concentration 0, 0.125, 0.25, 0.50, 0.75, and 1.0 mg/L, which validated the LC_{50} concentration under the experimental conditions.

2.2.1 Acute experiment of copper exposure

The acute experiments were conducted for two different groups for 96 h, one with copper LC_{50} concentrations and the other with the control group. Each experimental group contained 10 male crabs and the tanks were continuously aerated. The dissolved oxygen, temperature, pH and salinity were maintained the same as in the previous experiment. During the experiment, the crabs were fed *ad libitum*. The food debris and faeces were cleaned after 3–5 h of feeding.

2.3 Sample collection

After the acute exposure to copper three crabs from each group were randomly selected for further analysis. Haemolymph was extracted from the walking leg by using a 2.5 mL sterile syringe containing 1 mL of anticoagulant solution (30 mM trisodium citrate, 0.34 M sodium chloride, 10 mM EDTA, 0.115 M glucose, pH 7.5), and stored at 4 ºC. The haemolymph was centrifuged at 3500 rpm for 10 minutes at 4 ºC and the supernatant was collected and stored for further studies. The hepatopancreas, gills and muscle tissues were dissected immediately after cryo-anesthetized. The dissected parts were stored in 4% paraformaldehyde for histological studies and the remaining parts were used for the metal analysis and biochemical analysis (Fig. 1).

2.4 Copper accumulation analysis

The copper concentrations were analysed in different organs such as gills, hepatopancreas, and muscle of crabs by using atomic absorption spectroscopy (AAS) following the standard procedure (AOAC, 1995). Two crabs were selected randomly from each group and cryo-anesthetized and dissected samples were collected and prepared for metal analysis according to (Williams et al., 2022).

2.5 Estimation of biochemical components

The total protein was estimated by (Lowry et al., 1951) method. Briefly, 100 mg of crab tissue samples were homogenized in ethanol and centrifuged at 2000 rpm for 15 minutes. The resultant precipitate was dissolved in 1 mL of 1 N NaOH, 5 mL of alkaline copper solution, followed by 20 minutes of room temperature incubation. 0.5 mL of Folin- Ciocalteu phenol reagent was added and incubated for 20 minutes. The bovine serum albumin was used as a standard, and absorbance was measured at 650 nm against a blank, using a UV-visible spectrophotometer. The free amino acids in the crab tissues were determined according to (Moore and Stein, 1948) method. Homogenizing 500 mg of crab tissues with 2 mL of a mixture of sodium tungstate and 0.66 N of H_2SO_4 in a 1:1 ratio and centrifuged at 2000 rpm for 10 minutes. The fixed quantity of supernatant was mixed with distilled water and 4% ninhydrin reagent,

and placed in a water bath at 80 ºC. Absorbance was measured at 540 nm against blank and Leucine was used as a reference solution.

The total lipids were determined by the (Jordi Folch, M. Lees and Stanely, 2011) method. A mixture of chloroform and methanol in the ratio of 2:1 (V/V) is used to extract lipids from animal tissues. 0.9% NaCl was added to the resulting solution and kept overnight. The lipid layer was separated, dried and dissolved in H_2SO_4. The mixture was taken for a 10 minutes water bath and added sulpho-phospho-vanillin reagent after cooling. The mixture was shaken thoroughly and allowed to stand for 30 minutes. The colour intensity was taken at 520 nm using a UV-visible spectrophotometer. To find out the total amount of carbohydrates in the crab we used (Roe, 1955) method, the 500 mg homogenised tissues were centrifuged and added anthrone reagent the colour change was measured at 620 nm.

2.6 Analysis of antioxidant and metabolic enzymes

Tissue samples of crab from control and copper-treated were homogenized individually in ice-cold 50 mM tris and centrifuged (9000 g at 4 ºC for 20 minutes). The supernatant was used for testing antioxidant enzyme activity.

2.6.1 Catalase (CAT)

The CAT activity was measured using Sinha, (1972) method using hydrogen peroxidase as substrate and expressed as µmole of hydrogen peroxide decomposed per minute per mg protein. Briefly, 500 mg of tissues were homogenised in 1 mL of 0.01 M phosphate buffer (p H 7.1). The samples were centrifuged at 10,000 rpm for 10–15 minutes at 4ºC. To the 1 mL of enzyme extract (supernatant), 2 mL of 0.2 M hydrogen peroxide was added and mixed well. After 1 minute of incubation at room temperature 2 mL dichromatic-acetic acid mixture (1:3) was added. The tubes were placed in a boiling water bath for 10 minutes. After the colour formation cooled to room temperature and absorbance was measured at 570 nm against a blank.

2.6.2 Superoxide dismutase (SOD)

The autoxidation of pyrogallol in the tris buffer is the key principle of measuring the SOD activity levels in the samples. The SOD activity was analysed according Marklund and Marklund, (1974). Briefly, the 500 mg tissues were homogenised in 1 mL of 0.01M phosphate buffer (pH 7.1). Centrifuge the solution at 10,000 rpm for 15–20 minutes at 4ºC and collect the supernatant. To the test, 2mL of enzyme extract (supernatant), 2 mL of Tris-HCl buffer (pH 8.2, which also has 1 mM EDTA dissolved in it), and 0.5 mL of 2 mM pyrogallol were added, whereas in the control, distilled water was added instead of enzyme extract. Mix gently and immediately measure the absorbance at 430 nm per minute. SOD activity was expressed as unit per mg of protein.

2.6.3 Glutathione peroxidase (GPx)

GPx activity level was measured according to the Paglia Donald E, (1989) method, with cumene-hydroperoxide used as the substrate. Briefly, 1 mL of the reaction system containing 50 mM phosphate

buffer (pH 7.6), 30 mM glutathione, 4.5 mM Nicotinamide adenine dinucleotide phosphate (NADPH), 30 mM sodium azide, 10 U/mL glutathione reductase and 7.5 mM cumene hydroperoxide was added to an appropriate volume of tissue samples. The decrease in NADPH absorbance at 340 nm reflects the activity of GPx in the sample. All the reagents except the tissue sample were taken as a control.

2.6.4 Lipid peroxidation (LPO)

The LPO activity was measured for different tissues such as gills, hepatopancreas, and muscles, by estimating the thiobarbituric acid reactive substances according to Ohkawa et al., (1979) method. Tissues were homogenised in an ice-cold 0.01M phosphate buffer (pH 7.1). The homogenates were centrifuged at 10,000 rpm for 15–20 minutes at 4ºC and collect the supernatant. Briefly, 10% of trichloric acid (TCA), 0.02M Tris-HCl buffer (pH 7.5) and 1.5 mL of thiobarbituric acid (10%) (TBA) solution were added to 1 mL of enzyme extract (supernatant). The mixture was incubated for 15 minutes boiling water bath and immediately centrifuged at 100Xg for 20 minutes. The supernatant contains MDA-TBA complex, and the OD was measured for the supernatant at 535 nm. Along with this, a reagent blank was prepared except for the tissue homogenate. The colour intensity formed is proportional to the quantity of MDA formed, in turn indicating the LPO activity.

2.6.5 Serum Glutamic oxalic transaminase (SGOT) and Serum glutamic pyruvic transaminase (SGPT)

The metabolic enzymes glutamic pyruvic transaminase (GPT) and glutamic oxalic transaminase (GOT) activity in the serum were measured following the method of Reitman and Frankel (1957) .

The SGOT level is measured by adding the substrate solution, L-aspartic acid:α-ketoglutarate to a 100 µL serum and incubating for 1 h at 37ºC. After the incubation 0.5 mL of 2,4-dinitrophenyl hydrazine solution (DNPH) was added to this mixture and further incubated for 20 minutes at room temperature. Finally, 3 mL of freshly prepared 0.4 N NaOH was added and the absorbance was measured at 505 nm. Sodium pyruvate was used as a standard to measure the SGOT.

SGPT activity was estimated by adding 0.5 mL of substrate as L- alanine: α-ketoglutarate with 100 µL of serum and incubating for 20 minutes at 37ºC. Followed by adding 0.5 mL 2,4-dinitrophenyl hydrazine solution (DNPH) to this mixture and incubating at room temperature for 30 minutes. After the incubation 3 mL of 4 N NaOH was added. The colour intensity was measured at 505 nm and sodium pyruvate was used as a standard. The SGOT and SGPT were expressed as units per milligram of protein.

2.6.6 Acid phosphatase (ACP) and alkaline phosphatase (AKP) enzyme analysis

The ACP and AKP enzyme activities were evaluated according to the basic principle o (Andersch and Szcypinsk, 1947), where P-nitrophenol phosphate was used as a reagent substrate. ACP or AKP hydrolyses phenyl phosphate to phenol and inorganic phosphate. The ACP and AKP experiments were conducted as per the instructions followed by Coral Clinical Systems (India), cat no: 1102010102.

ACP activity was estimated by adding 0.1 mL of haemolymph was mixed with 1 mL of working reagents (kit provided). The working reagent contains citrate buffer (pH 5) provides optimum acidic conditions, and the colouring reagent, 4-aminoantipyrine (0.6%) and potassium ferricyanide (2%), along with trichloroacetic acid (TCA). The absorbance was measured after 25–30 minutes of incubation at room temperature. The ACP hydrolyses the phenol in an acidic medium and the free phenol reacts with 4-aminoantipyrine and potassium ferricyanide to form red coloured solution. The absorbance measurements were taken repeatedly at 1,2, and 3-minute intervals. The enzyme activity was calculated as the unit of phosphatase released per g protein in the haemolymph.

AKP activity was estimated by the phenol release under the alkaline condition (pH 10). The working reagents contain glycine-NaOH buffer (pH 10) provides optimum alkaline conditions, and the colouring reagent, 4-aminoantipyrine (0.6%) and potassium ferricyanide (2%), along with trichloroacetic acid (TCA). Briefly, 20 µL of haemolymph sample was added to 1 mL of working reagents (kit provided) and mixed thoroughly. The reaction mixture was incubated for 15–20 minutes at room temperature. The absorbance was measured at 405 nm using a UV-visible spectrophotometer. The AKP activity was calculated by the unit of phosphates liberated per g protein in the haemolymph.

2.7 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein separation in the haemolymph of both the copper-treated and control groups was performed using SDS-PAGE according to Laemmli, (1970) and slightly modified as described by Albaige et al., (2001). Briefly, protein samples were mixed with 2X loading buffer (0.15 M Tris HCl, 1.2% SDS(w/v), 60% glycerol (v/v), 15% 2-mercaptoethanol (v/v), and 0.09% bromophenol blue (w/v) and boiled at 95 ºC for 3–5 minutes before running electrophoresis. 10 µg of protein samples were loaded into a 12% SDS gel at respective wells. The electrophoresis was run at 90 V for 1 h. After electrophoresis, the gels were stained with Coomassie Brilliant blue and washed with deionised water before being examined through a gel imager. Experiments were performed in triplicate.

2.8 Genotoxicity (Comet assay)

The DNA damage in haemocytes of both control and copper-treated groups was assessed according to (Singh et al., 1988) method with slight modifications as described in (Mayekar et al., 2012). 10 µL haemocyte suspension of both control and copper-treated was mixed with 90 µL of 0.6% agarose in phosphate-buffered saline (PBS) and immediately spread into microscope slides precoated with 1% agarose in PBS. After 15 minutes of solidification at 4 ºC, the slides were dipped in freshly prepared ice-cold lysis solution (1% sodium N-lauroyl sarcosinate, 2.5 M sodium chloride, 100 mM Ethylenediaminetetraacetic acid (EDTA), 10 mM Tris-HCl (pH 10), 1% Triton X-100 and 10% DMSO) for 2 h at 4 ºC. Followed by placing these slides in an electrophoresis tray with fresh-cold alkaline buffer (300 mM sodium hydroxide, 1 mM EDTA, at pH 3) for 20 minutes to unwind of DNA. Electrophoresis was performed for 30 minutes at 25 V and 300 mA (Power case, Life Technology). Finally, the slides were dipped in 0.4 M Tris-HCl buffer (pH 7.5) for the neutralisation of the alkali followed by staining with 50 µL

ethidium bromide (20 µg/mL). After 5 minutes of incubation, the slides were washed with PBS to remove the excess stain and slides were analysed using an inverted epifluorescent microscope Olympus CKX41 equipped with an Optika Pro % CCD camera. Comets were scored for both samples using Tritek Comet Scoring Software. Experiments were performed in triplicate.

2.9 Histopathological studies

After the copper treatment, the dissected gills, hepatopancreas and muscle samples of both the control and treated groups of *S. serrata* were preserved by using 4% paraformaldehyde. For routine histopathology, the standard procedure was followed sections of 4–5 µm were cut using a rotary microtome and stained with haematoxylin and eosin and mounted with DPX (Sigma, Aldrich). Observations were recorded and digital images were captured using a microscope Nikon Eclipse Ci.

2.10 Statistical analysis

All experiments were conducted in triplicate and the data were expressed as mean ± standard deviation. The significant difference between the control and treatment groups (of each tissue) of each parameter was calculated by using the independent-samples T-test. The significant difference among the tested groups of each variable was analysed by One-way analysis of variance (ANOVA) and followed by a homogeneity test of variances, and Duncan's multiple range test (DMRT). The significant difference was calculated at $p < 0.05$. All the analyses were conducted using the IBM SPSS (Version 20) software.

3 Results

3.1 Acute toxicity bioassay

The 96 h LC_{50} value of copper to *S. serrata* was 0.52 mg/L, as determined by probit analysis, and was statistically significant ($p < 0.05$). The slope of the line ($r^2 = 0.977$) indicates the moderately strong sensitivity of *S. serrata* to copper (Fig. 2).

3.2 Effect of copper bioaccumulation

The copper bioaccumulation in gills, hepatopancreas and muscles were analysed and recorded as 1.006 ± 0.083 mg/kg/wet wt, 0.845 ± 0.015 mg/kg/wet wt and 0.570 ± 0.018 mg/kg/wet wt respectively (Table. 1). The copper bioaccumulation in copper-treated gills was statistically significant when compared with control ($p < 0.05$). In the present study, the accumulation of copper in gills was found to be significantly higher compared with hepatopancreas and muscles.

3.3 Biochemical parameters

The biochemical constituents in living organisms play a vital role in physiological functions. The concentrations of total amino acids and lipids in the hepatopancreas of crabs were 1.84 ± 0.26 mg/g tissue and 3.93 ± 0.015 mg/g tissue respectively, which shows a statistically significant difference compared to the control groups ($p < 0.01$). However, the treated muscle and gills did not show any

significant difference in amino acid and lipid concentration. The carbohydrate and protein levels in the copper-treated serum were 5.25 ± 0.07 mg/g and 9.55 ± 0.005 mg/ml respectively, which is statistically significant compared to the control ($p < 0.01$) (Fig. 3). Among all biochemical parameters, the total protein content was significantly higher in the copper-treated tissues than in the control groups. Moreover, carbohydrate levels in copper-treated crabs were found to be significantly elevated than control crabs ($p < 0.05$).

3.4 Antioxidant enzymatic assays

In the current study, the antioxidant enzymes SOD, CAT, LPO, and GPx levels were significantly elevated in copper-treated crabs compared to control crabs, which indicates the oxidative stress faced by the organisms even under short-term copper exposure. As shown in Fig. 4, the level of CAT enzymes in the copper-treated gills (0.650 ± 0.10 U/mg) also significantly increased when compared with the control ($p < 0.05$). When compared across the different tissues of copper-treated crabs, the CAT activity levels show significant differences among them ($p < 0.05$). The SOD activity in the muscle exhibited a significant elevation from 2.71 ± 0.05 U/mg in the control to 4.62 ± 0.02 U/mg in the copper-treated group ($p < 0.01$). Compared to the different tissues of copper-treated the SOD levels show significant differences among them ($p < 0.05$). Among all the tissues examined, hepatopancreas showed the highest GPx activity, significantly elevated from 0.36 ± 0.04 U/mg to 0.62 ± 0.02 U/mg in the copper-treated group ($p < 0.01$). When compared across the tissues of copper-treated crabs, GPx levels show a notable difference ($p < 0.05$). In hepatopancreas, the LPO level rises from 0.243 ± 0.032 nmol MDA released/mg in the control to 0.53 ± 0.04 nmol MDA released/mg in the copper-treated group ($p < 0.01$). Notably significant differences were found when compared across the different tissues of the copper-treated crab ($p < 0.05$) (Fig. 4).

As shown in Fig. 5, the GOT and GPT levels in the serum of control and copper-treated crabs were analysed. An elevation of GOT in copper-treated serum was observed as 10.78 ± 0.49 in copper-treated crabs which is significant compared to the control value of 0.72 ± 0.25 ($p < 0.01$) (Fig. 5a). Whereas the SGPT levels in the serum were found to significantly increase in the copper-treated group to 11.571 ± 0.2050 U/mg when compared to their control, 4.8 ± 0.53 ($p < 0.01$) (Fig. 5b).

3.5 ACP and AKP enzyme studies

The ACP activity in the haemolymph of copper-treated crabs (5.13 ± 0.01U/mg) was increased significantly as compared to the control (2.59 ± 0.02 U/mg), which was statistically significant ($p < 0.05$) (Fig. 6). The AKP activity in the haemolymph of copper-treated crabs (27.42 ± 0.43 U/mg) was found to be significantly higher than the control (12.39 ± 0.22 U/mg) ($p < 0.05$).

3.6 SDS PAGE

As shown in Fig. 7 the protein bands were separated based on their molecular weight for the haemolymph of *S. serrata* of the control and copper-treated groups. Approximately 10 bands were

expressed according to molecular weight for both groups from 10 kDa and 250 kDa. A high intensity band was expressed at 70 kDa in the haemolymph of the copper-treated group of *S. serrata* compared to the control group (Fig. 7).

3.7 The single-cell gel electrophoresis assay (comet assay)

The genotoxicity effects of copper in *S. serrata* at acute exposure were evaluated in the comet assay, where haemocytes were used to examine the extent of DNA damage (Fig. 8). As shown in Table. 2, copper caused a significant increase in the tail DNA percentage, indicating the extent of single strand DNA breaks. The high percentage of tail length indicates that copper accumulation in haemocytes showed a simultaneous genotoxic response.

3.8 Histological studies in gills, hepatopancreas and muscles

Acute exposure to copper leads to structural alterations at the cellular level in different organs of *S. serrata*. The effect of copper on the structure of the gill in *S. serrata* was studied. The longitudinal section of the branchial stem of *S. serrata* gills exhibited normal architecture compared to copper-treated crabs (Fig. 9a-b). The primary and secondary lamellae were arranged well and a layer of cuticles and a thin layer of epithelial cells were present. The lamellae were arranged uniformly with uniform lamellar space and normal haemocoel space with optimum haemocytes. In addition, their gill tips have keratinous epidermis. The nuclei, pillar cells and haemocytes show normal architecture of gills. In contrast to the control crabs (Fig. 9c-d), major alterations were observed in the copper-treated crab's gill architecture. The distorted and necrotic secondary gill lamellae and deformed epithelium of the gill lamellae. The external keratinous epidermis of the gill tips was swollen and broken. Fractured or broken basement membranes were present.

In the control crabs, it shows the normal architecture of the hepatopancreas of many decapod species is shown with tightly packed columnar cells. Uniformly and evenly distributed haemocyte cells, normal nuclei and, normal tubular architecture. The B and R cells appeared in all tubes, and the star-shaped lumina were present (Fig. 10a). However, the sections of the hepatopancreas in the copper-treated crab showed the presence of more vacuolized cells and the internal organization of the lumen was severely damaged and loss of the star-shaped lumen. Moreover, there appeared the haemolytic infiltrations in the interstitial sinuses of the hepatopancreas and the presence of more R cells. Cellular aggregation accumulates in the epithelial lumen. Necrotic cells and tissue debris were observed. The thickened and detached basal lamina with necrotic and swollen cells (Fig. 10b-d).

Sectioning through the muscle tissue of *S. serrata* control group revealed normal muscle architecture. The fascicular arrangement of muscle fibres, the muscle bundles and the presence of uniform connective tissues are the indications of the normal structure of muscle (Fig. 11a). In contrast, the muscle section of the copper-treated group of *S. serrata* was completely altered with haemocyte infiltrations and condensations of connective tissue elements into heavily stained eosinophilic materials.

The muscle bundles show severe myositis, haemocytes cellular infiltrations and a severe cluster of nuclei (Fig. 11b).

4 Discussion

Heavy metal pollution has been a major concern and threatens aquaculture because of its non-degradability, toxicity and persistence (Paez-Osuna, 2001). Copper is one of the toxic heavy metals present in the aquaculture, sand mining, and agricultural runoff, causing serious threats to aquatic organisms at excess concentrations (Qian et al., 2020). Copper metal is a key ingredient in algicides and fungicides profoundly used in aquaculture farms and as an essential mineral in the diet feed (Yeh et al., 2004). Although copper is an essential element for crustaceans and participates in various biological and immunological functions (Velayutham and Munusamy, 2016), the excess copper concentrations may cause negative impacts on crustaceans health (Chen and Lin, 2001).

This study explored the impact of acute exposure to copper on the biochemical, antioxidant and metabolic enzyme systems and histopathological alterations in *S. serrata*. Many studies have been conducted about the acute copper exposure in crustaceans over the years. The LC_{50} value of copper in the shoe crab *Carninus maena* was 0.5 mg/L (Truchot and Rtal, 1998) and the LC_{50} of copper in *Potamonautes warren* was 1.0 mg/L (Vosloo et al., 2002). Moreover, the LC_{50} concentration of copper chloride in crab *Sesarma quadratum* was 28 ppm (Santhana Valarmathi, 2002), 6 ppm in the *Portunus pelagicus* under higher salinity (Ketpadung et al., 2006). However, in this study the LC_{50} concentration of copper in *S. serrata* was recorded as 0.52 mg/L. Compared to previous studies, it's evident that the LC_{50} of copper varies among the different crab species (Fig. 2). Therefore, monitoring the copper concentration among crab species is necessary to mitigate its negative impacts on the crab’s health and its wild population.

Although excessive copper concentrations could bioaccumulate and may affect the normal physiological functions of gills such as respiration, excretion and osmoregulation even during short term exposure (Martinez et al., 2011; Ren and Pan, 2014; Weihrauch et al., 2001). Previously, the copper bioaccumulation in crabs gills was found to be higher compared to muscles and hepatopancreas upon copper-treated crabs (Arockia Vasanthi et al., 2014). Metal accumulation is higher in the gills because gill tissues often come into contact with the outer environment and serve as one of the major pathways for metal entry into the organisms (Xu et al., 2018). Since the hepatopancreas is the main organ for detoxification and biotransformation in crustaceans and is more sensitive to heavy metal pollution than muscles (Ortega et al., 2017; Ren and Pan, 2014). These results altogether along with the present study, show that the copper bioaccumulation was higher in the gills compared to the hepatopancreas and muscle tissues (Table 1). The distribution of metals in tissues portrays the way of accumulation and the resulting amount of accumulation is determined by the detoxification routes of the organisms (Rainbow and Black, 2005).

Table 1
Bioaccumulation of heavy metal copper (mg/kg/wet wt) in different tissues of mud crab *S. serrata*

Experimental Condition	Samples Tested (Mg/Kg/ wet wt)		
	Gills	Hepatopancreas	Muscles
Control	0.753 ± 0.010	0.737 ± 0.024	0.545 ± 0.026
Test	$1.006 \pm 0.083^{a*}$	$0.845 \pm 0.015^{b*}$	0.570 ± 0.018^{c}

Each value is the mean ± SD of three replicates. Mean values sharing different alphabetic letter superscripts among tested tissues are statistically significant at $p < 0.05$ (One way ANOVA and Duncan's multiple range test were used), * - indicates the significant difference between the control and test of each tissue, where $p < 0.05$, (independent-samples T-test)

Table 2
The comet length and comet tail length of control and copper-treated *S. serrata* at 96 hours of exposure

Sample	Comet Length (px)	Comet Tail (px)
Control	42 ± 0.394	0.888 ± 0.098
Test	$70.37 \pm 4.716^{*}$	$6 \pm 1.492^{*}$

Each value is the mean ± SD of three replicates, * - The difference between the control and treated gills is statistically significant ($p < 0.05$), (Single tail T-Test)

Excessive copper exposure may threat to normal physiology and biochemical processes of aquatic animals, especially crustaceans. Among the biochemical parameters evaluated, the total protein content was significantly higher in the copper-treated groups than in the control groups. Since the enhanced protein synthesis may need to meet the energy demand for the damaged tissues and heightened immune response as a means to combat metal toxicity in their tissues at short-term exposure (Barathkumar et al., 2022). The enhancements in the LPO imply the oxidative damage in the liver, hepatopancreas, possibly due to insufficient antioxidant production to inhibit tissue damage. In parallel to the histological studies in hepatopancreas, copper stress causes cell membrane degeneration, liver dysfunction and disturbance of lipid metabolism and ultimately it favors elevations of total lipid components (Latif et al., 2014). The elevated carbohydrate levels serve as an instant energy source during acute toxic conditions to withstand low energy levels under heavy metal stress (Javed et al., 2017). Lipids and carbohydrates are closely related to the nutritional metabolism and immunological regulations in aquatic animals (Dong et al., 2018; Zuo et al., 2023). Under stress conditions, crabs need more energy to detoxify the toxicants to minimize adverse metal exposure (Xuan et al., 2011). The high protein levels would increase the protease activity in hepatopancreas and muscles, which in turn increases amino acid levels as well. The results clearly show a significant elevation in the biochemical parameters observed in the copper-treated crabs upon acute exposure in response to the crabs undergoing a detoxification process which required more energy demands (Xuan et al., 2011).

The antioxidants enzymes are tracked for the toxicity assessments since oxidative stress is stand as the primary indicator in crustaceans (Kumar et al., 2024). The antioxidant enzymes SOD, CAT, LPO, and GPx levels gets significantly elevated in copper-treated crabs tissues compared to control crabs, which indicates the oxidative stress faced by the organisms under short-term copper exposure (Fig. 3). Previous reports have shown that *S. serrata* samples from heavy metal polluted sites show high antioxidant enzyme activity (Yogeshwaran et al., 2020). Multiple studies were reported the elevation of antioxidant and metabolic enzymes have been observed across various crustaceans upon heavy metal exposure such as *Cyprinus carpio* sampled from a polluted environment (Karadag hasan, Ozgur Firat, 2014), estuarine crab *Neohelice granulate*, (Sabatini et al., 2009), white shrimp *Litopeneaus vannameii* (Guo et al., 2015)d *vannameii* (Betancor et al., 2021) suggesting polluted environment triggers a common stress mechanism. GPT and GOT are part of protein synthesis as transaminase but the enhanced level of GOT and GPT in serum is generally accompanied by parallel histological damages in the hepatopancreas.

It was also notable that the higher ACP and AKP level in the acute copper-treated crabs shows the indication of oxidation stress and damage faced by the organisms. The elevation of ACP enzyme reflects lysosomal membrane damage caused by oxidative stress due to heavy metal toxicity (Sang et al., 2023). The elevated AKP level is linked to the structural damage to the tissues such as gills, hepatopancreas, and muscles (Saha et al., 2009). The elevated antioxidants and metabolic enzymes are used as biomarkers in ecotoxicological studies to assess the toxic index of the habitat.

All these biochemical and enzymatic results clearly indicate that whether it is copper exposure in laboratory conditions or waterborne copper exposure in an open environment, the metal toxicity and the metal resistance may vary with species, but all the biochemical reactions to lessen the heavy metal toxicity inside the organisms were found to be similar in all species. The SGOT and SGPT are part of protein synthesis as transaminases but the enhanced level of GOT and GPT in serum is generally accompanied by parallel histological damages in the hepatopancreas. In turn, it reflects the physiological stress in the organisms and causes oxidative damage upon copper exposure.

Interestingly, the above results reveal that the acute exposure triggers the sudden antioxidant response by enhancing the antioxidant enzymes due to ROS overload and the shock response. In addition, Table 3 shows the biomarker response of acute copper exposure in the different crab species, which reveals the elevation of antioxidant enzymes to cope with the oxidative stress induced by copper metal toxicity. In contrast, the chronological studies of copper in crab species in (Bao et al., 2020) (Bu et al., 2022) and the ammonium by (Neil et al., 2005) state that the long term metal exposure poses a gradual accumulation that leads to a decline in antioxidant and metabolic enzymes activities and fails to induce metabolic shifts and eventually causes irreversible damage in the tissues and organs. By comparing our study with previous chronic studies highlight the sudden triggered enzymatic responses upon acute copper toxicity and differentiates the acute oxidative stress compensation with chronic physiological permanent damages in crab species, especially in *S. serrata*.

Table 3. Biomarker responses to the copper acute studies in various crab species in the previous studies

Species	Exposure type	Exposure days	Copper concentrations	Biomarkers	References
Callinectes sapidus	Acute	48 h	0.75mg/L	SOD, GPx ↑	(Marius Brouwer, 1998)
Eriocheir sinensis larvae	Acute	96 h	0.21mg/L	SOD, CAT, LPO, GST ↑	(Sun et al., 2014)
Pachygrapsus marmoratus	Acute	48 h	0.52mg/L	SOD, CAT, ChEs, GR ↑	(Oliva et al., 2019)
Eriocheir sinensis	Acute	5 days	0.04mg/L 0.18mg/L 0.70mg/L	SOD, GST, MDA ↑	(Feng et al., 2022)
Scylla serrata	**Acute**	**96 h**	**0.5mg/L**	**SOD, CAT, LPO, SGOT, SGPT ↑**	**Present study**

The protein profiling in the copper-treated crab serum revealed a prominent band at 70 kDa in corresponds to the molecular weight of Hsp70, a heat shock protein found to be upregulated upon metal exposure. Although not confirmed by western blotting, this band may indicate Hsp70, as mentioned in previous studies in crustaceans upon metal stress. Hsp70 was found to be significantly upregulated with 7.5 fold in *Artema sinica* (Qian et al., 2010). Similarly, the Hsp70 protein was highly expressed in bluegill sunfish collected from a polluted area (Yoo and Janz, 2003). Indeed, the molecular chaperone Hsp70 has the function of repairing protein damage caused by heavy metal exposure. The prominent protein bands at 70kDa indicate the animal under undergone oxidative stress and their immunological functions have been activated to mitigate the copper induced stress faced by the organisms.

For better information about the genotoxicity effects of copper in *S. serrata* upon acute copper exposure, the haemocytes were used in the comet assay to examine the extent of DNA damage (Fig. 8). As shown in Table. 2, copper caused a significant increase in the tail DNA percentage, indicating the extent of single strand DNA breaks. The failure of antioxidant defence to reduce the ROSs produced by copper exposure could lead to oxidative damage to DNA. The higher concentrations of copper in a particular tissue lead to enhanced ROS production and consequently cause DNA strands break (Kumar et al., 2024). Our study stated a significant correlation exists between genotoxicity and copper accumulation (Al-Subiai et al., 2011).

Further, acute copper exposure leads to structural alterations at cellular levels, distorted and necrotic secondary lamellae, and deformed epithelium of gill lamellae were found in the gills of the mud crab *S.*

serrata. Previously, the heavy metal exposure disrupted the membrane (Revathi et al., 2011), thickening of the basal lamina (Yu et al., 2023), and an increased number of R cells (Li et al., 2007) in the hepatopancreas of different crustaceans were observed. The cellular damage in the muscle tissues of crustaceans is mainly due to lipid peroxidation in the membrane and protein damage (Ridgway et al., 2007). It is also noted that the oxidative stress in blue crab *Callinectes amnicolas* causes muscle degeneration and necrotic muscle fibres with haemorrhage (Chukwuka et al., 2023). These previous reports support our present investigation of the changes in the normal architecture of muscle upon acute exposure to copper in *S. serrata*, which altered their normal functioning indicating that acute exposure to copper could bring oxidative stress and even oxidative damage in the organisms, suggests that long term exposure of copper may leads to the life threatens of the animal. The histological analysis showed that gill tissues exhibited more prominent alterations compared to minor changes in the hepatopancreas and muscles, due to their frequent contact with the metal contaminated environment.

The oxidative stress triggers a sudden elevation of oxidative and metabolic enzymes, eventually followed by oxidative damage causing cellular damage in organs. This suggests that the copper induced oxidative damage at a point overwhelms the crab's detoxification capacity, resulting the minor cellular damage in the tissues. Although copper toxicity enhances the activity of antioxidants such as SOD, CAT, LPO, and GPx, the antioxidant response remains insufficient to overcome the oxidant damage caused by copper exposure (Qian et al., 2020). The significantly enhanced metabolic enzymes in serum further indicate the stress and damage in the hepatopancreas and muscles. Such metabolic disturbance may directly impact the aquaculture productivity by compromising the growth, molting, and reproductive viability of *S. serrata*.

In addition, *S. serrata* exhibits a notable potential recovery mechanism upon copper toxicity. The elevation of antioxidant enzymes as the primary defense to reduce the oxidative stress and the increased biochemical parameters to survive at the critical stage of toxicity reflects the recovery potential of *S. serrata*. Additionally, the prominent protein bands corresponding to the Hsp70 indicate a molecular level protective attempt to restore cellular repairs and homeostasis. The elevated lipid peroxidation and the DNA damage in the comet assay results further confirm the oxidative stress as the primary cause. These mechanisms of *S. serrata* show its response against acute copper exposure. However, it seems that longer exposure may surpass these recovery mechanisms and the organisms may suffer permanent tissue damage and impaired physiological functions.

In this study, we discussed the toxic effects of copper to *S. serrata* at their LC_{50} concentrations. Comparing these LC_{50} concentrations from our study with real-world levels, especially in India, enhances the ecological relevance of our findings. Over the years, there are several studies have been done to assess the copper metal concentration in soils, water resources and the coastal regions across the India (Alexander and Thomas, 2011). For instance, a study conducted in agricultural soil at the Singhbhum shear zone, India, reported the presence of a wide range of copper metal (47 ppm to 299 ppm) (Giri et al., 2017). Similarly, copper metal research was conducted in the Yamuna River water were found to be exceeding the permissible limits to WHO, implying a hazardous risk to human health (Parihar et al.,

2021). These findings suggest the chance for higher copper levels in the environment may vary in seasons, climate, and the influence of anthropogenic activities. Therefore, it advocates the necessity of monitoring heavy metals in natural habitats.

5 Conclusion

In conclusion, the heavy metal copper exposure on *S. serrata* causes multilevel impacts including biochemical and physiological aspects and histological alterations. In the present study, we found that the elevated levels of antioxidant and metabolic enzymes in *S. serrata* evidenced the copper stress faced by the organisms. The relationship between oxidative stress with gills, hepatopancreas and muscles were investigated in this study, which provides the irreversible severe necrotic lamellae, thickened basal laminae, the formation of vacuoles, and the heavily stained eosinophilic substances respectively, which all are indicating the importance of considering the oxidative damages at cellular levels caused by copper under short-term exposure. The observed biochemical, enzymatic, and genotoxic disruptions could negatively affect survival rates and growth rates, leading to economic losses for farmers. In this context of emerging heavy metal pollution in aquaculture systems these findings emphasize the need for regulations and monitoring of copper concentrations in aquaculture environments. While the cellular effects are characterized, underlying molecular pathways warrant further investigation, hence, it requires detailed analysis to understand the influence of copper toxicity on physiological and molecular aspects.

Declarations

Consent to participate

The authors declare their Consent to participate in this article.

Consent to publish

The authors declare their Consent to publish this article.

Ethical approval

Not applicable. All applicable international/national or institutional guidelines for the care and use of animals were followed.

Credit authorship contribution statement

A.A: Conceptualization, Methodology, Formal analysis, Investigation, Validation, Resources, Original manuscript writing, writing- review and editing. **J.P**: Data curation, Formal analysis, writing- review and editing, **A.Ak**: Data curation, Formal analysis, writing- review and editing, **M.V**: Supervision, Original manuscript writing, Conceptualization, Methodology, Formal analysis, writing - review and editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

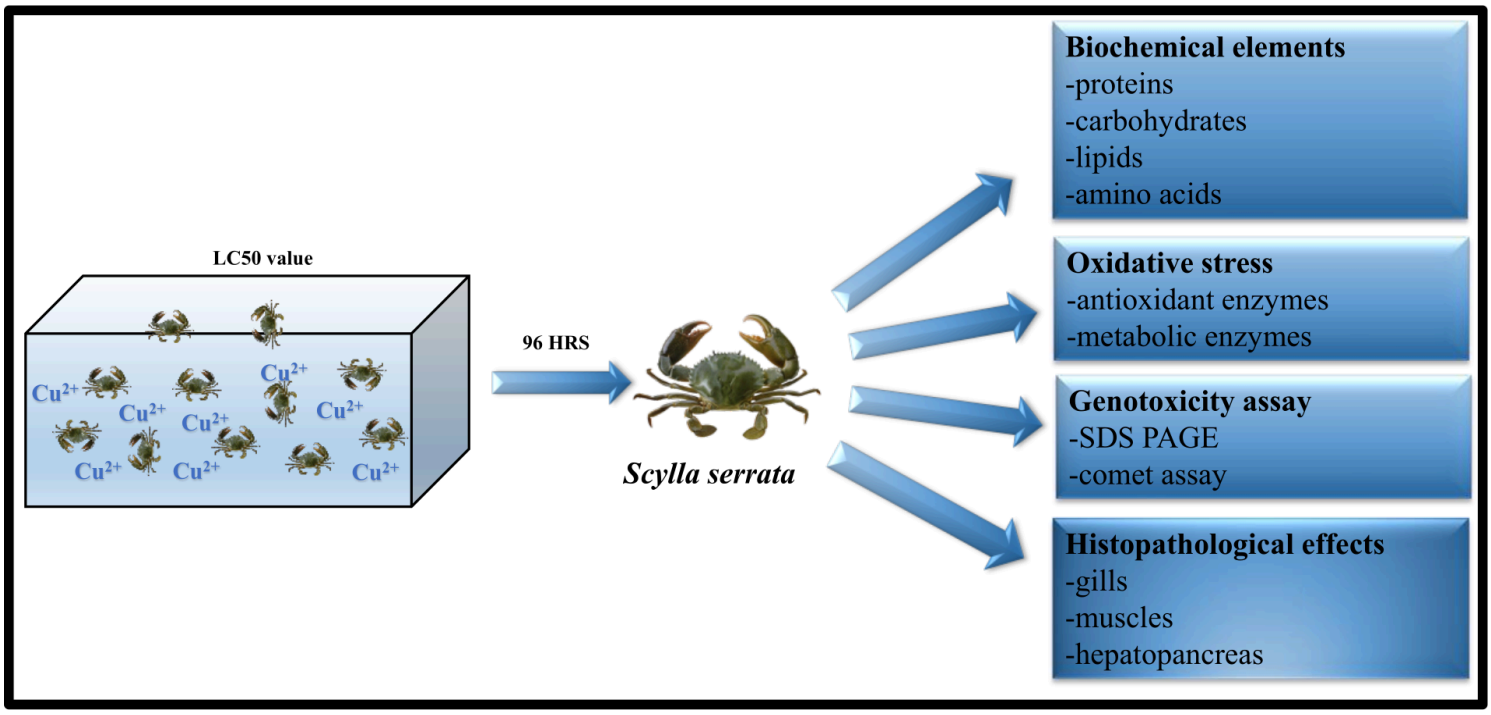


Figure 1

Schematic representation of the effects of acute copper toxicity exposure to *Scylla serrata* at 96 hours.

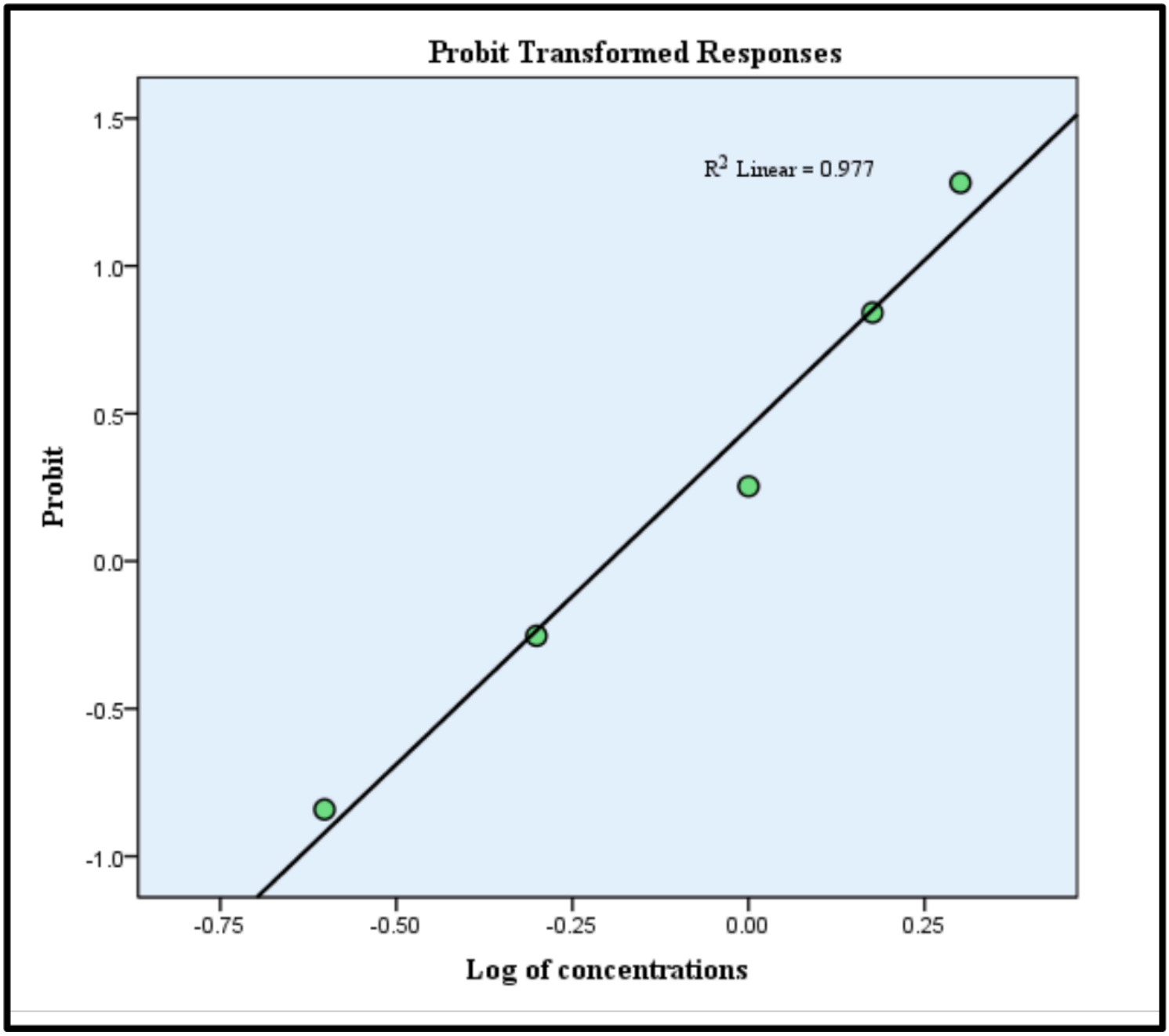


Figure 2

LC_{50}- Probit regression graph of 96h of copper exposure on *Scylla serrata.*

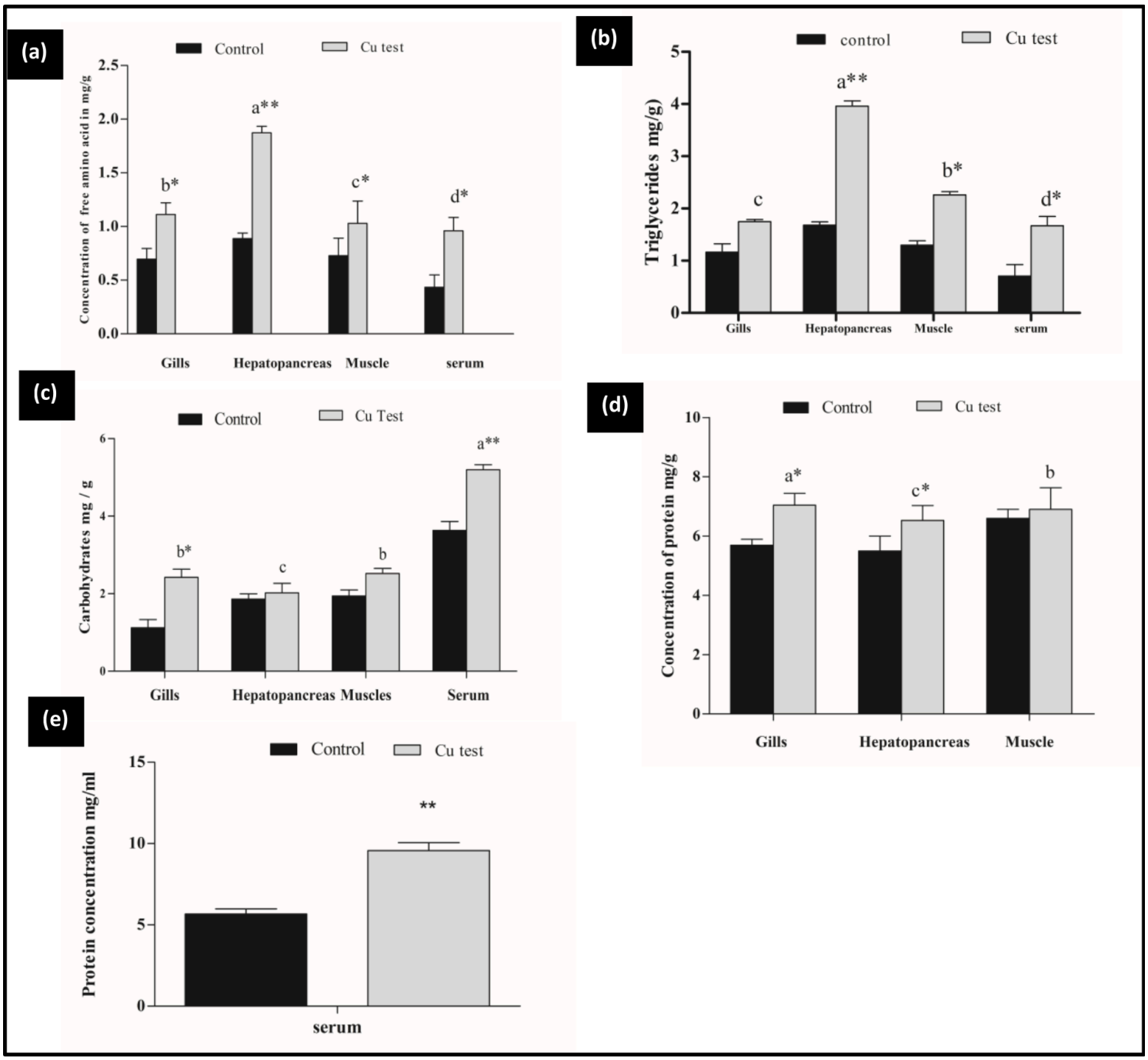


Figure 3

Effects of LC_{50} copper concentrations on biochemical constituents in the different tissues of *S. serrata*. a – amino acids, b – lipids, c – carbohydrates, d – protein, e – serum protein. Each value is the mean ± SD of three replicates. The bars are represented as Mean±SD of three replicates. Different alphabetical letters indicate that there is a significant difference between the tested groups of each tissue (p <0.05). * - Indicate the statistical significance between the control and test of each tissue. The increase in value between the control and copper-exposed group is statistically significant (*p<0.05, ** p <0.01).

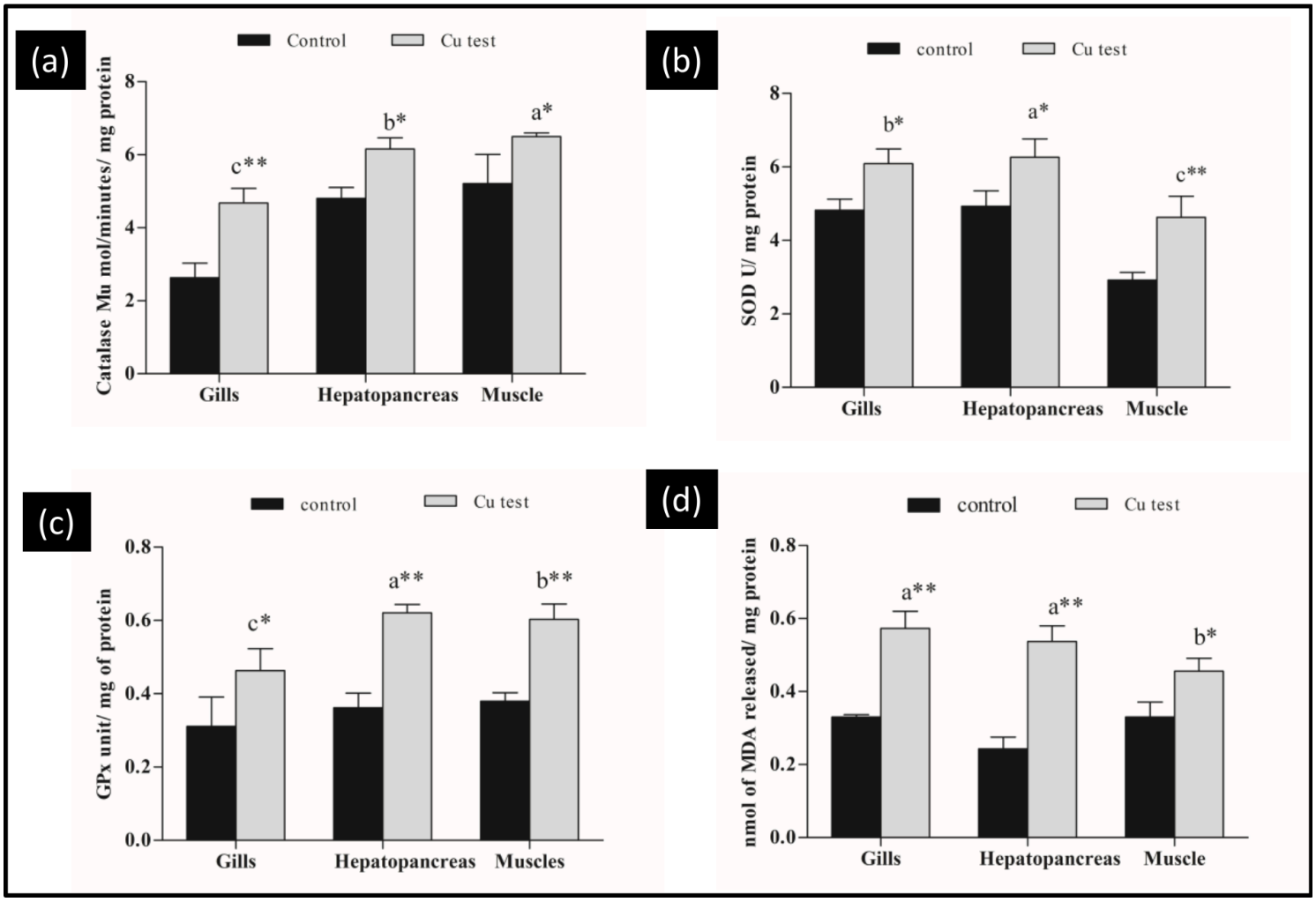


Figure 4

Effects of LC_{50} copper concentrations on antioxidant enzymes in the different tissues of *S. serrata.* a - CAT, b - SOD, c - GPx, d - LPO. Effects of LC_{50} concentrations of copper on antioxidant enzyme activities. The bar is presented as mean±SD of three replicates. Different alphabetical letters indicate that there is a significant difference between the tested groups of each tissue, (p <0.05). * - Indicate the statistical significance between the control and test of each tissue. The increase in value between the control and copper-exposed group is statistically significant (*p<0.05, ** p <0.01).

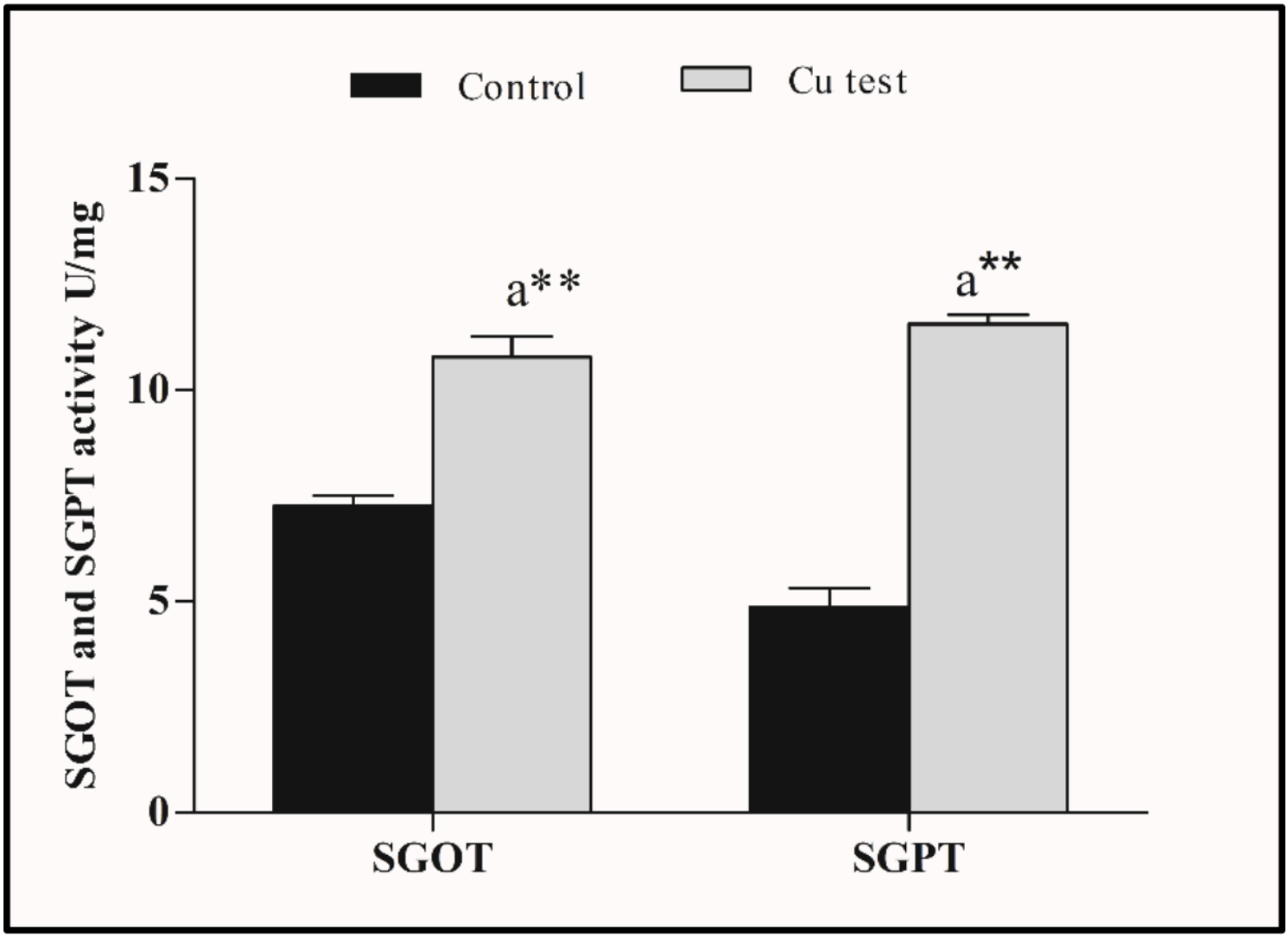


Figure 5

Effects of LC_{50} concentrations of copper on metabolic enzymes GOT and GPT activities in serum, presented as U/mg protein. The bar is presented as mean ± SD, n=3, * - indicates a significant difference between treatments (p <0.01).

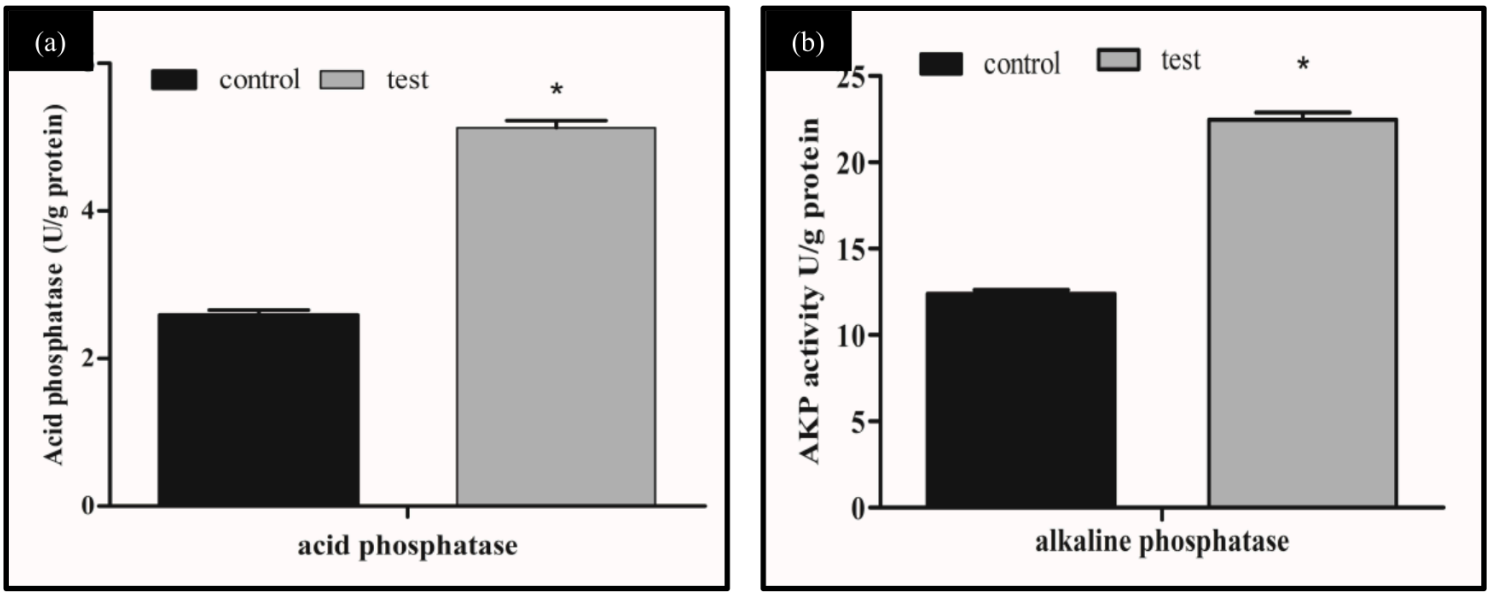


Figure 6

Effects of LC_{50} concentrations of copper on (a) - acid phosphatase (ACP) and (b) - alkaline phosphatase (AKP) activities in serum, presented as Units/g protein. The bar is presented as mean± SD of three replicates, * - indicates the significance at $p<0.05$.

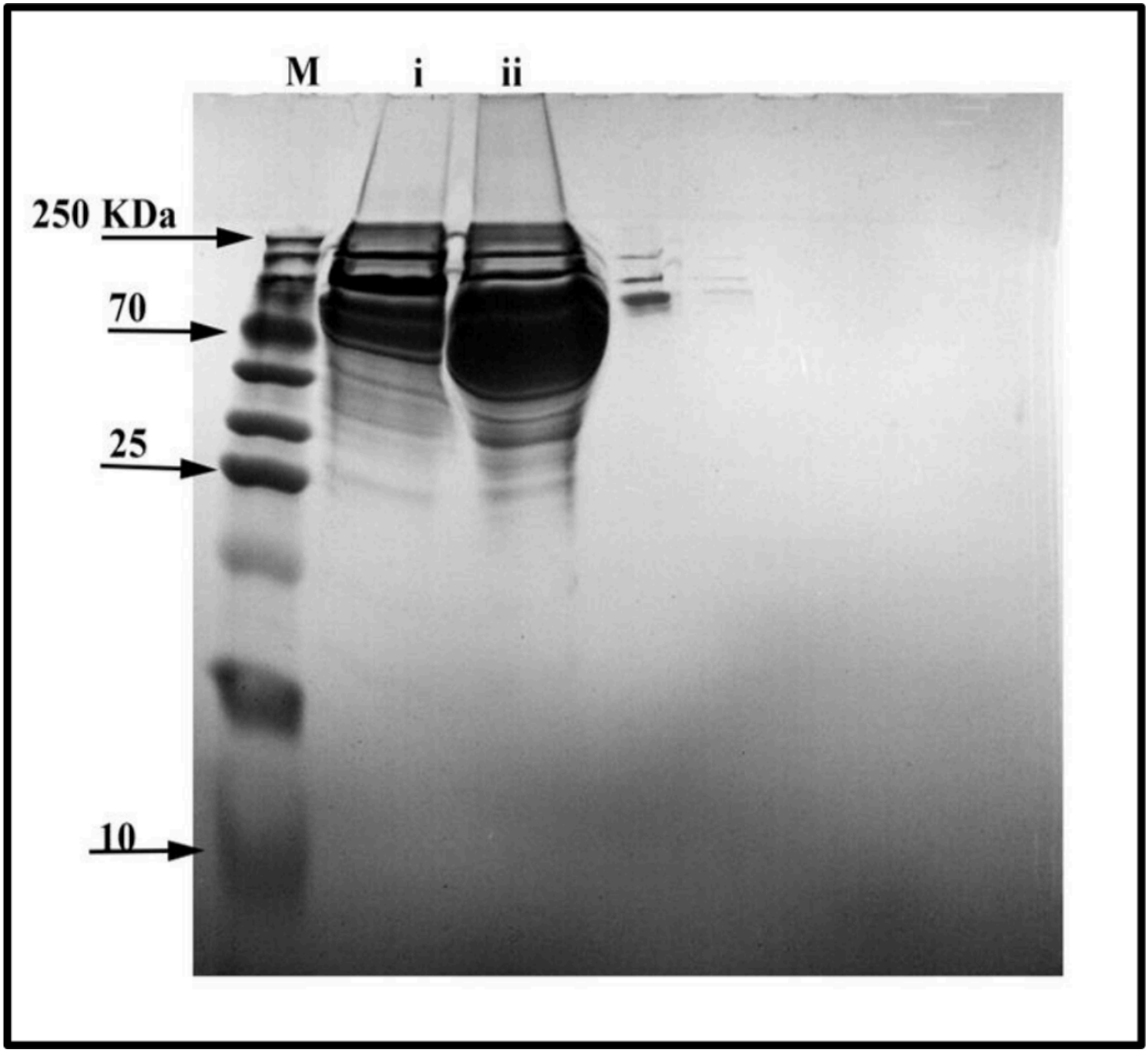


Figure 7

Shows the protein profiling of haemolymph M- marker, i- control and ii- copper-treated

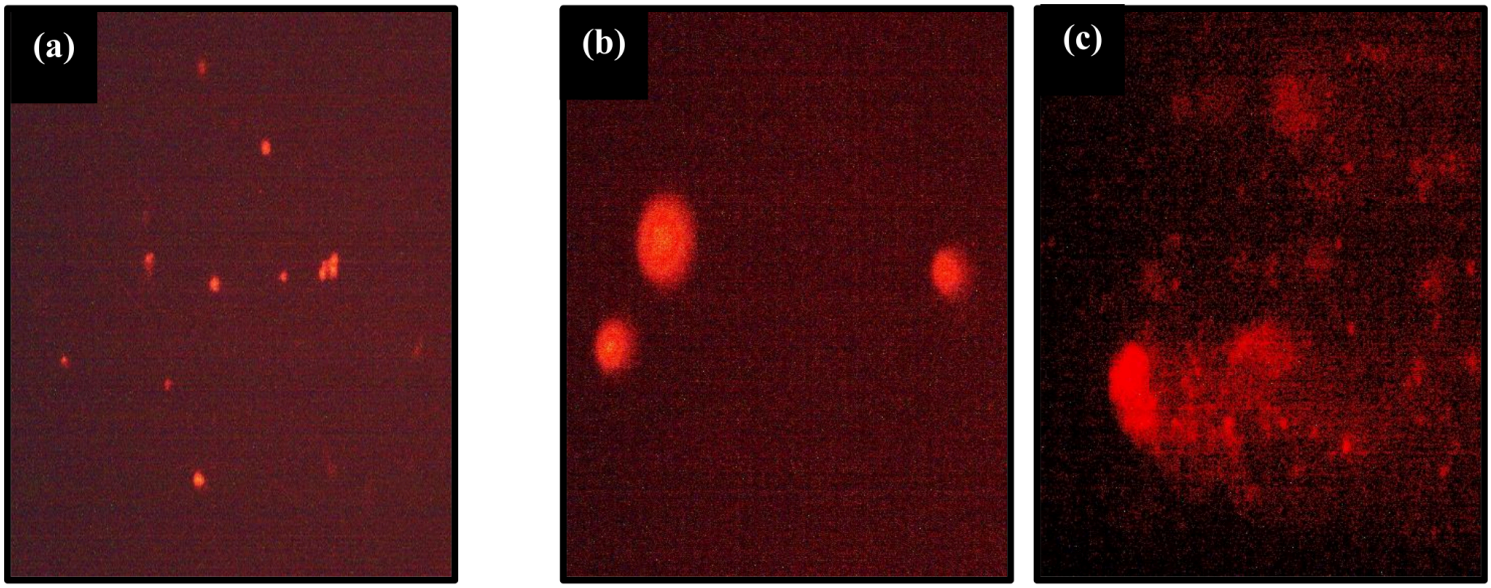


Figure 8

Grades of DNA tail in hemocytes of *Scylla serrata* the control hemocytes - a and the copper-treated hemocytes b-c.

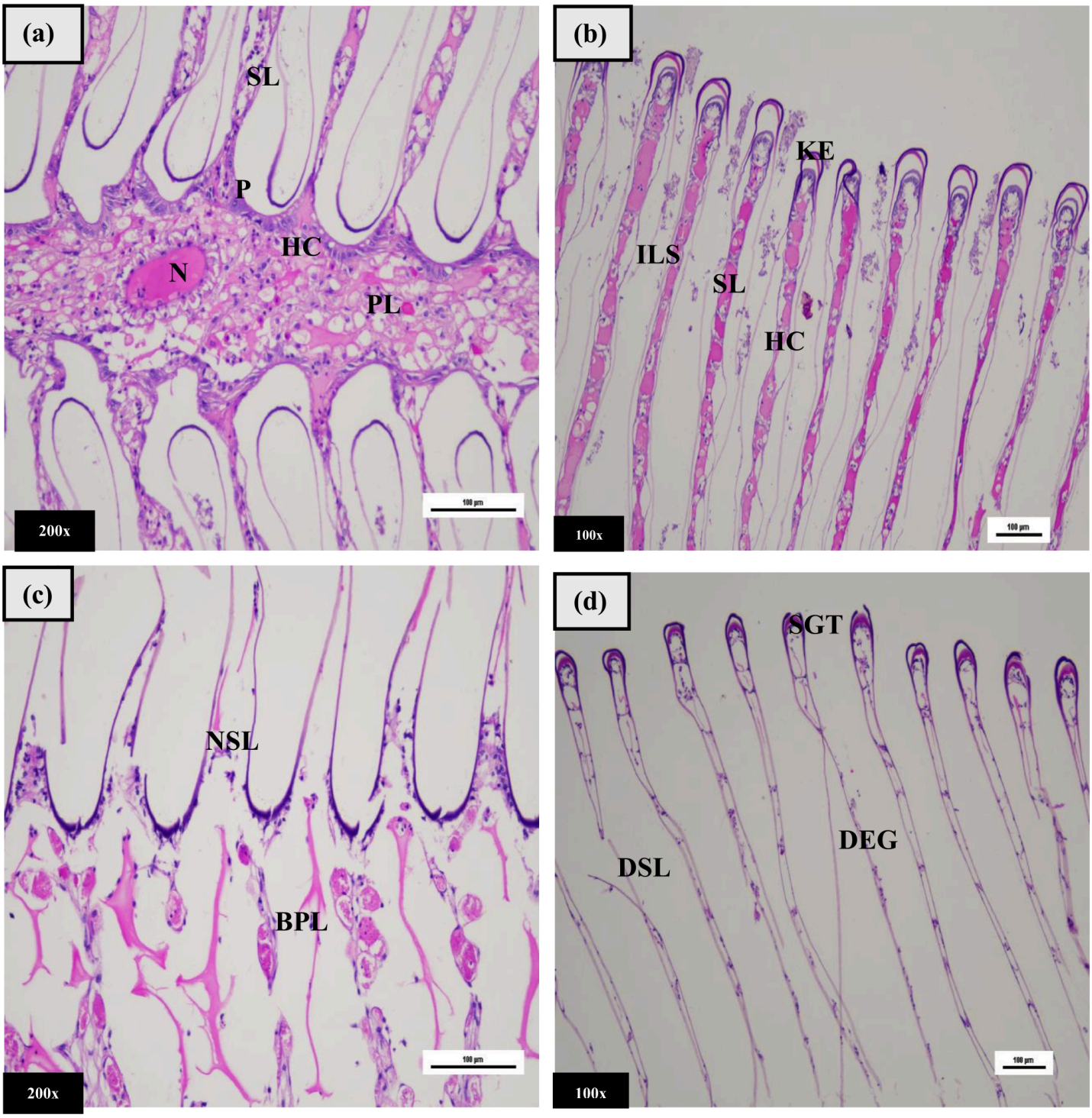


Figure 9

Photo micrograph of gills of *S. serrata* stained with haematoxylin and eosin. a–b Sections of control gills. Showing well-arranged primary lamellae (**PL**), secondary lamellae (**SL**), pillar cells (**P**), haemocytes (**HC**), Normal nuclei (**N**), uniformly arranged haemocytes and pillar cells, Keratinous epidermis (**KE**), uniform intercellular lamellar space (**ILS**). c-d Sections of treated gills. Swollen gills tips (**SGT**) Distorted secondary lamellae (**DSL**), necrotic gill lamellae (**NGL**), deformed epithelium of gill lamellae (**DEG**).

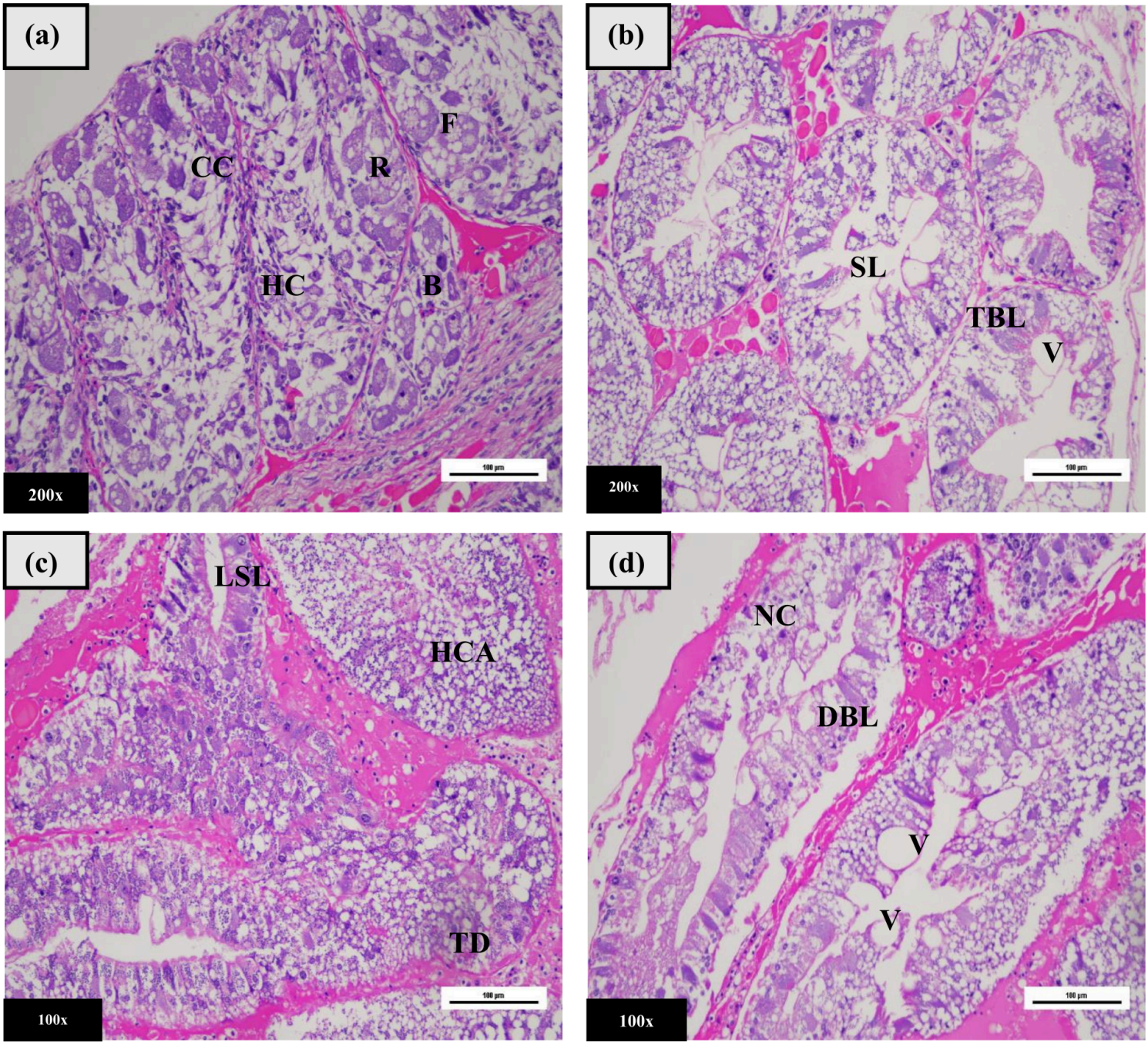


Figure 10

Photomicrograph of a section of the hepatopancreas of *S. serrata* stained with haematoxylin, and eosin. a – control hepatopancreas showing the normal architecture of hepatopancreas, tightly packed columnar cells (**CC**), Uniformly and evenly distributed haemocyte cells (**HC**), normal nuclei and, normal tubular architecture, presence of R cells (**R**), B cells (**B**), and F cells (**F**) are appeared in all tubes. b - d Sections of hepatopancreas in test crab after 96 hr of copper exposure showing, vacuolized cells (**V**), and haemolytic infiltrations (**HI**) in the interstitial sinuses of the hepatopancreas. Severely damaged lumen and loss of star-shaped lumen (**LSL**), haemolytic cellular aggregation (**HCA**). Presence of necrotic cells (**NC**) and tissue debris (**TD**). The thickened basal lamina (**TBL**) and detached basal lamina (**DBL**).

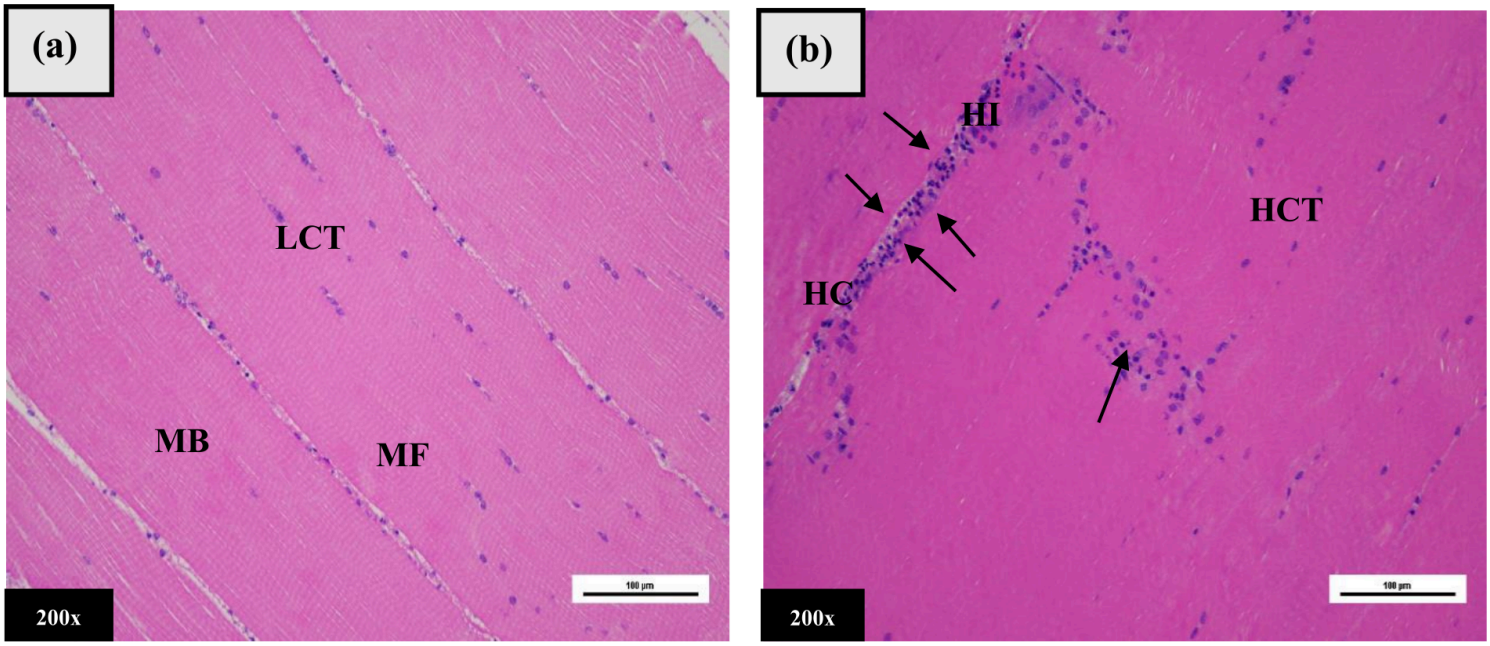


Figure 11

Photomicrograph of a section of muscles of *S. serrata* stained with haematoxylin, and eosin. a **-** control muscle showing normal muscle pathology, Normal arrangement of muscle fibres (**MF**), muscle bundles (**MB**), and Uniform light connective tissues (**LCT**). b **-** Test muscles showing Haemocyte infiltration (**HC**), condensation of connective tissue elements into heavily stained eosinophilic materials (**HCT**), Severe myositis, haemocyte cellular infiltration (**HI**), and a severe cluster of nuclei.